



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of

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Serial No. 09/596,444

Examiner Ann Y. Lam

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Group Art Unit 1641

For : PHOSPHORYLATION ASSAYS

U.S. Patent and Trademark Office
Attention: Ann Y. Lam
Group Art Unit 1641
Washington, D.C. 20231

Sir:

DECLARATION OF J. RICHARD SPORTSMAN UNDER 37 C.F.R. § 1.132

I, J. RICHARD SPORTSMAN, declare:

1. I am one of the inventors named on U.S. Patent Application Serial No. 09/596,444 ("the '444 application").

2. I am currently an employee of Molecular Devices Corporation ("MDC"), which is the assignee of the '444 application. I currently hold the position of Vice President, Assay and Reagent Research and Development, at MDC. If called as a witness, I could competently testify to the following facts, all of which are within my own personal knowledge.

General Background

3. I am trained as an analytical biochemist. I received a Ph.D. in Analytical Chemistry from the University of Arizona in 1982, and I completed post-doctoral studies in Clinical Immunology at Scripps Clinic and Research Foundation in 1984.

4. I have worked at several biotechnology or pharmaceutical companies, including Eli Lilly and Company, SyStemix, Inc., and Terrapin Technologies (now Telik). Each of these companies is or was involved in drug discovery or development of human therapeutics. In my work at these companies, I have acquired special expertise in methods of drug discovery using binding tests ("assays") based on fluorescence polarization.

5. I have been employed by MDC or LJJ Biosystems ("LJJ") since August 1998. In August 1998, I joined LJJ, which merged with MDC two years later in August 2000. At LJJ and MDC, I have been responsible for research on various chemical tests and the development of kits that use such tests.

6. Further details of my employment history and qualifications are described in my resume, which is attached as an Appendix of this Declaration.

Comparison of Iron and Gallium in Binding Assays

7. I designed and conducted a series of binding assays to compare the metals iron ("iron reagent") and gallium ("gallium reagent"). Each binding assay included either a fluorescent substrate (a nonphosphorylated peptide) or a fluorescent product (a phosphorylated form of the peptide). After incubation of the fluorescent substrate or product with the iron reagent or the gallium reagent, I measured the effect of each metal on luminescence intensity and polarization from the substrate or product. Accordingly, these binding assays with gallium correspond to an embodiment of the invention for detecting kinase enzyme activity in which there is no enzyme activity (substrate (S) only) or substantial enzyme activity (product (P) only). Alternatively, by reversing the substrate and product designations of these peptides, the results correspond to an

embodiment of the invention for detecting phosphatase enzyme activity in which there is substantial enzyme activity or no enzyme activity, with similar conclusions.

I. Reagents

8. I used the following reagents for the binding assays. Binding buffer was prepared as an aqueous solution of 50 mM acetic acid and 500 mM NaCl, titrated to pH 5.0 with NaOH. Concentrated stock solutions of the metals were prepared as 0.67M FeCl₃ or 0.67M GaCl₃ in 0.1 M HCl. The iron reagent and gallium reagent were prepared for addition to binding assays by 400-fold dilution of each respective, concentrated metal stock solution with the binding buffer. Reaction buffer (10 mM Tris-HCl, 10 mM MgCl₂, 0.1% BSA, 0.05% NaN₃, pH 7.2) was used to prepare 100 nM solutions of fluorescent peptides for the binding assays.

9. The fluorescent peptides used for the binding assays have amino acid sequences corresponding to a fragment of glycogen synthase. These peptides were synthesized chemically and have the following sequences: (1) 5FAM-Lys-Lys-Leu-Asn-Arg-Thr-Leu-Ser-Val-Ala (substrate, "S"), and (2) 5FAM-Lys-Lys-Leu-Asn-Arg-pThr-Leu-Ser-Val-Ala (product, "P"). In these sequences, "5FAM" represents a 5-carboxy fluorescein moiety (a luminophore) covalently linked to the amino terminus of each peptide, and pThr is phosphothreonine. The substrate (S) and product (P) peptides differ only by the absence (S) and presence (P) of a phosphate group on threonine. The substrate peptide has been used successfully in kits sold by Molecular Devices Corporation to test the activity of MAPKAP K2, PRAK, and CaM KII enzymes using the gallium reagent in fluorescence polarization assays.

II. Performance of Binding Assays

10. Binding assays were performed using the reagents described above in Section I. Binding reactions were assembled by combining 60 μL of the iron reagent or the gallium reagent with 20 μL of 100 nM S or P peptide in wells of a microplate. Each binding reaction was incubated for thirty minutes at room temperature. Then, fluorescence polarization was measured on an Analyst $\text{\textcircled{R}}$ AD instrument, which is available commercially from Molecular Devices Corporation. In particular, each binding reaction was illuminated with polarized light of 490 nm, and component fluorescence intensities were measured from directions parallel (I_{\parallel}) and perpendicular (I_{\perp}) to the direction of polarization for the incident polarized light.

11. Fluorescence polarization (FP) was calculated according to the following formula, where G is an instrument factor:

$$\text{FP (in mP)} = 1000 \times (I_{\parallel} - G \times I_{\perp}) / (I_{\parallel} + G \times I_{\perp})$$

Total fluorescence intensity (I_{TOT}) was calculated according to the formula:

$$I_{\text{TOT}} = I_{\parallel} + 2 \times I_{\perp}$$

Each combination of reaction components was assayed in triplicate, and standard deviations were calculated based on these triplicate assays.

III. Results of Binding Assays

12. Figure 1 shows results of total fluorescence intensity measurements in relative fluorescence units (RFU), as a function of metal (iron reagent or gallium reagent) and peptide phosphorylation state (P or S) in the binding assays.

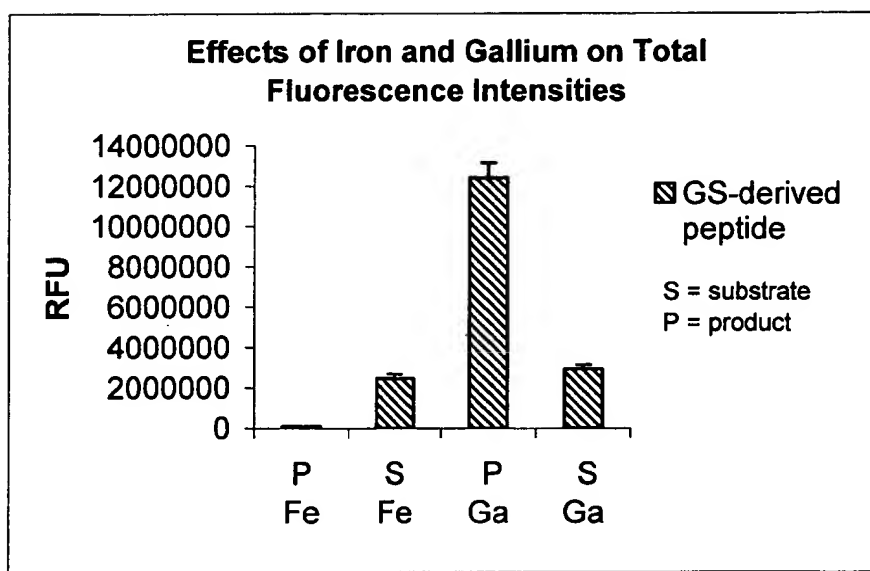


Figure 1

The fluorescence intensity was generally comparable in assays of the substrate (S) performed with either the iron reagent or the gallium reagent. This result is consistent with little or no binding of each reagent to this substrate, and thus little or no effect on the brightness of the luminophore associated with this substrate. However, the fluorescence intensity or brightness differed dramatically according to added metal (iron or gallium) in assays of the product (P). In particular, the brightness was about one-hundred-fold higher in assays of the product performed with the gallium reagent relative to the iron reagent. This difference in brightness was produced by an approximately four-fold increase in the brightness of the product relative to substrate for the gallium reagent, and by a more than twenty-fold decrease in this brightness relative to substrate (S) for the iron reagent. Therefore, the iron reagent quenched the brightness of the product substantially, while the gallium reagent not only did not quench but actually enhanced this brightness.

13. Figure 2 shows a plot of fluorescence polarization (mP) measured as a function of added metal and peptide phosphorylation state.

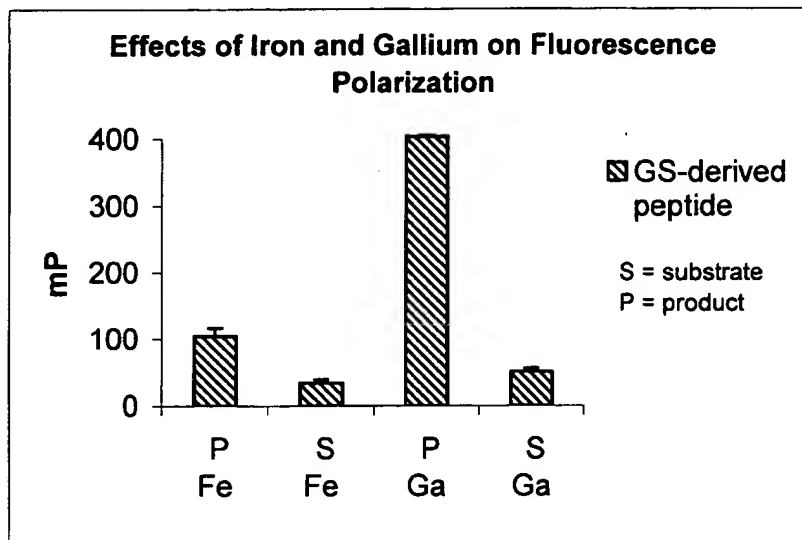


Figure 2

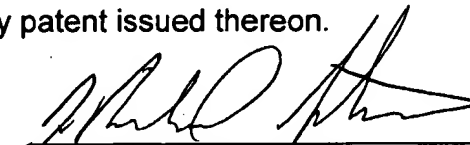
The difference between the degree of polarization produced by luminophore-associated product and substrate defines the "dynamic range" of these assays. In the present configuration, the iron reagent provides a dynamic range of about 70 mP, and the gallium reagent a dynamic range of about 350 mP.

Statement Under 18 U.S.C. §1001

14. I hereby declare that all statements made herein of my own knowledge are true and that statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of title 18 of the United State Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: _____

4-4-06


J. RICHARD SPORTSMAN